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Dysregulation of serine biosynthesis contributes to the growth defect of a *Mycobacterium tuberculosis* *crp* mutant

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Summary

Mycobacterium tuberculosis CRP_{Mt}, encoded by Rv3676 (*crp*), is a CRP-like transcription factor that binds with the *serC*–Rv0885 intergenic region. In the present study, we evaluated CRP_{Mt}'s regulation of *serC* and Rv0885 in *M. tuberculosis* and *M. bovis* BCG, using site-specific mutagenesis, promoter fusions and RT-PCR. The CRP_{Mt} binding site was required for full expression of *serC* and Rv0885, and expression of both genes was reduced in *M. tuberculosis* and *M. bovis* BCG *crp* mutants. These data show that CRP_{Mt} binding directly activates both *serC* and Rv0885 expression. *M. tuberculosis* *serC* restored the ability of an *Escherichia coli* *serC* mutant to grow in serine-dropout medium, demonstrating that *M. tuberculosis* *serC* encodes a phosphoserine aminotransferase. Serine supplementation, or overexpression of *serC*, accelerated the growth of *M. tuberculosis* and *M. bovis* BCG *crp* mutants in mycomedium, but not within macrophages. These results establish a role for CRP_{Mt} in the regulation of amino acid biosynthesis, and show that reduced serine production contributes to the slow-growth phenotype of *M. tuberculosis* and *M. bovis* BCG *crp* mutants in vitro. Restoration of serine biosynthesis by *serC* expression will facilitate identification of additional CRP_{Mt}-regulated factors required by *M. tuberculosis* during macrophage and host infection.

Introduction

Tuberculosis (TB) remains a global epidemic, with nine million new cases and two million deaths annually, and one-third of the world population latently infected. Drug resistance and a lethal synergy between the causative agent *Mycobacterium tuberculosis* and human immunodeficiency virus (HIV) are major causes of this high morbidity and mortality (http://www.who.int/tb/publications/global_report/2009/update/en/index.html). Gene regulation is critical for *M. tuberculosis*'s successful response to the diverse environments encountered within the host during infection. However, little is known about gene regulatory mechanisms in *M. tuberculosis*.

Adenosine 3', 5'-cyclic monophosphate (cAMP) is an important signaling molecule in diverse organisms, including prokaryotic and eukaryotic microbes as well as their mammalian hosts. The classical prokaryotic cAMP signaling pathway established in *Escherichia coli* for catabolite repression requires interaction of cAMP with the cAMP receptor protein (CRP) (Kolb *et al.*, 1993). However, cAMP's broader roles in regulating metabolism and pathogenesis of many pathogens are being increasingly recognized

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(Alspaugh *et al.*, 2002, Caler *et al.*, 2000, D'Souza & Heitman, 2001, Gross *et al.*, 2003, Petersen & Young, 2002, Wolfgang *et al.*, 2003). CRP homologs in *Pseudomonas aeruginosa*, *Yersinia pestis* and *Salmonella enterica* serovar Typhimurium coordinate expression of a range of virulence factors, including type IV pili and the type III secretion system (TTSS) (Whitchurch *et al.*, 2005, Miao *et al.*, 1999, Petersen & Young, 2002). Mutation of the adenyl cyclase-encoding gene (*cya*) in *Vibrio vulnificus* caused attenuation in a murine infection model (Kim *et al.*, 2005), and a cAMP- dependent Cl⁻ secretory pathway is associated with the diarrhea induced by *Aeromonas sobria* (Tanoue *et al.*, 2005). Pathogenic fungi use the highly conserved cAMP signal transduction pathway to regulate cellular differentiation as well as their virulence potential (Pukkila-Worley & Alspaugh, 2004), and the cAMP-PKA signal transduction pathway is required for *Aspergillus fumigatus* pathogenicity (Liebmann *et al.*, 2003, Liebmann *et al.*, 2004).

Recent work shows that *M. tuberculosis* increases cAMP production upon phagocytosis by macrophages (Agarwal *et al.*, 2009, Bai *et al.*, 2009), suggesting that cAMP signaling is important for *M. tuberculosis* pathogenesis. This finding is consistent with results of an early study showing that infection of macrophages with *M. microti* elevated cAMP levels and impaired phagosome-lysosome fusion in the infected cells (Lowrie *et al.*, 1975). Genes have been identified for at least 15 adenyl cyclases in *M. tuberculosis*, and activity has been confirmed for the products of at least 10 of these genes (Castro *et al.*, 2005, Guo *et al.*, 2001, Linder *et al.*, 2002, Reddy *et al.*, 2001, Shenoy & Visweswariah, 2006a, Sinha *et al.*, 2005, Tews *et al.*, 2005, Abdel Motaal *et al.*, 2006). The further presence of 10 genes encoding putative cNMP-binding proteins suggests a wide range of functional roles for cAMP in *M. tuberculosis* biology (McCue *et al.*, 2000, Shenoy & Visweswariah, 2006b).

CRP_{Mt} (Rv3676) is one of two cAMP-associated transcription factors identified in *M. tuberculosis* (McCue *et al.*, 2000, Gazdik *et al.*, 2009, Gazdik & McDonough, 2005). CRP_{Mt} is a CRP-like protein that binds a DNA motif resembling *E. coli* CRP's DNA binding site (Bai *et al.*, 2005, Rickman *et al.*, 2005, Spreadbury *et al.*, 2005). Two amino acid differences in *M. bovis* BCG's CRP_{Mt} ortholog (CRP_{BCG}) increase its DNA binding affinity and have some effects on gene expression, but no significant functional or virulence-related differences between the two proteins have been found (Bai *et al.*, 2007, Hunt *et al.*, 2008, Stapleton *et al.*, 2010).

We previously identified a putative regulon for CRP_{Mt} comprising 114 genes (Bai *et al.*, 2005), and additional regulon members were proposed in a recent study (Krawczyk *et al.*, 2009). However, CRP_{Mt}'s direct role in regulation has not been established for most of the above genes. The putative CRP_{Mt} regulon includes *serC*, which has been identified as an essential gene in *M. tuberculosis* (Sasseti *et al.*, 2003). In *E. coli*, *serC* encodes a phosphoserine aminotransferase that is essential for growth in the absence of serine supplementation (Clarke *et al.*, 1973). An *M. tuberculosis crp* deletion strain showed impaired growth in bone marrow-derived macrophages and in a mouse model of TB (Rickman *et al.*, 2005). Growth was also reduced in vitro, but the bases of these growth defects are not known. We reasoned that decreased expression of *serC* could contribute to the reduced growth rate of the *M. tuberculosis crp* mutant.

In this study, we used *serC* and its divergently transcribed gene, Rv0885, to study CRP_{Mt}'s role in regulation of gene expression. We found that expression of both genes is directly activated by CRP_{Mt}'s binding to its predicted binding site. We also identified the gene product of *serC* in *M. tuberculosis* (and in *M. bovis* BCG) as a phosphoserine aminotransferase involved in the synthesis of serine. In addition, we showed that serine supplementation increases the *crp* mutants' growth in vitro, but not within macrophages.

These studies establish a role for CRP_{Mt} in the regulation of amino acid biosynthesis in *M. tuberculosis* and provide mechanistic insights into that regulation.

Results

Mapping the 5' ends of the *serC* and Rv0885 transcripts

The upstream intergenic region and open reading frame (orf) sequences of *serC* and Rv0885 are identical in *M. bovis* BCG and *M. tuberculosis*, with the exception of a single base change that results in a T234I difference in the *M. bovis* BCG Rv0885 ortholog, relative to that of *M. tuberculosis*. The transcription start sites (TSSs) of *serC* and Rv0885 were determined by primer extension, using total RNA isolated from exponentially growing *M. bovis* BCG. The TSS of Rv0885 mapped to a G, which is 84 nt from the translation start codon (Fig. 1A). A potential Pribnow (−10) box (AAGGGT) with 3-of-6 bp match to the consensus sequence TAYGAT (where Y is either a C or a T) (Mulder *et al.*, 1997) is centered at a suboptimal 7 nt upstream of the Rv0885's TSS, while the CRP-binding motif is centered at −55.5 nt from this TSS.

Several possible TSSs for *serC* were detected with oligonucleotides KM1608 and KM1609, as we have observed for some other mycobacterial genes (Vasudeva-Rao & McDonough, 2008). Use of KM1609 mapped one potential TSS to a G located 103 nt upstream from the start codon (Fig. 1B). The CRP-binding motif is centered at +21.5 nt from this *serC* TSS, while a potential −10 region (TATGAC) with a 5-of-6-bp match with the consensus sequence (TAYGAT) is centered at −13 nt. A second possible TSS (Fig. 1C) was also mapped using oligonucleotide KM1608, which is 70 nt from the start codon and 13.5 nt downstream of the CRP binding motif axis. An additional faint band of greater size was also observed with each primer. The TSSs associated with these faint bands are separated by 6 nt and flank the divergent Rv0885 TSS. RT-PCR was performed using a series of primers (Table 2) stepping progressively upstream through this region to confirm the presence of the longer extension products. Product was obtained with all primers except one that binds a region beyond the upstreammost site identified by primer extension (Fig. S1). While these results validate the upstream TSSs, differences in the efficiencies of the different primers prevent us from drawing conclusions regarding the actual relative use of each possible TSS.

The CRP_{Mt}-binding motif is important for expression of *serC* and Rv0885 promoters

We previously identified a CRP_{Mt} binding motif in the *serC*-Rv0885 intergenic region (Bai *et al.*, 2007, Bai *et al.*, 2005) (Fig. 1D). In the present study, we constructed promoter:*lacZ* reporter fusions for *serC* and Rv0885 using their intergenic DNA region to evaluate the role of CRP_{Mt} in regulation of these two genes. We measured the activities of both promoters in *M. bovis* BCG, given that the *serC*-Rv0885 intergenic DNA binds CRP_{Mt} and CRP_{BCG} similarly (Bai *et al.*, 2007).

Truncated reporter fusions of *serC* and Rv0885 promoters that excluded DNA sequences upstream of the CRP_{Mt}-binding motif ('long' fragments) had 50% (*serC*) to 33% (Rv0885) of the activity of their respective full length promoters in *M. bovis* BCG (Fig. 2). The decreased promoter activities indicate that DNA sequences upstream of the CRP_{Mt} binding site contribute to expression of both genes. However, truncation beyond the CRP_{Mt}-binding motif ('short' fragments) completely abolished the expression of both promoters (Fig. 2), demonstrating the importance of the CRP_{Mt}-binding site for the expression from either promoter.

We replaced 16 bp of the motif core (TGTGANNNNNTCACA) with a 16 bp random sequence (AGGCTTTCGTTCCGCT) to more specifically test the role of the CRP_{Mt} binding site. Constructs in which the same DNA sequence was used to replace regions 18 bp

upstream or downstream of the motif were included as controls (Fig. 3A). The activities of the mutant promoters were compared with WT, in *M. bovis* BCG. CRP_{Mt} motif replacement alone reduced the expression of each promoter ~5-fold (Fig. 3B), consistent with the promoter truncation results. As expected, substitution of DNA sequences in the 'Ctl 2' region (Fig. 1D, 3A) had no effect on either promoter's activity (Fig. 3B). However, the 'Ctl 1' DNA replacement (Fig. 1D, 3A) increased expression ~5-fold for *serC*, and ~3-fold for Rv0885, compared with the WT promoters.

The increased expression associated with the 'Ctl 1' replacement suggested that the modified DNA sequence either enhanced the binding with CRP_{Mt}, or reduced binding of an unknown repressor for both *serC* and Rv0885. To determine whether the CRP_{Mt} binding was altered by this sequence substitution, we labeled the WT and mutant promoter regions as probes, and used EMSA to assess CRP_{Mt}'s binding with each DNA sequence. Replacement of the binding site totally abolished the binding with CRP_{Mt} (Fig. 3C), while both substitution control fragments retained their binding with CRP_{Mt} at levels similar to those of the WT promoter sequences (Fig. 3C). The same results were observed using CRP_{BCG} (data not shown). We conclude that the 'Ctl 1' region contains a binding site for an unidentified repressor (Fig. 3A).

The role of the CRP_{Mt} binding site in *serC* and Rv0885 regulation was further defined with point mutations. We previously showed that nt G4 and C17 in the CRP_{Mt} motif model NNTGTGANNNNNTCACANN are highly conserved, and are crucial for CRP_{Mt}-binding to the *serC*-Rv0885 intergenic region (Bai *et al.*, 2005). CRP_{BCG} produced the same result (Fig. 4A), so we generated *serC* and Rv0885 promoter reporters with G4 and C17 point mutations (Fig. 4B), and measured their expression levels in *M. bovis* BCG. Expression of these mutated promoters was reduced ~2-fold for *serC* and ~3-fold for Rv0885, compared with the WT levels (Fig. 4C). These results indicate that the altered nucleotides are required for full activity of the promoters, further suggesting that CRP_{Mt} positively regulates expression of both *serC* and Rv0885.

Expression of *serC* and Rv0885 in *M. tuberculosis* and *M. bovis* BCG *crp* mutants

M. tuberculosis crp and *M. bovis* BCG mutants were generated by homologous recombination (Fig. 5A) to establish the role of CRP_{Mt} (or CRP_{BCG}) itself in expression of *serC* and Rv0885. The replacement of *crp* with a hygromycin resistance marker in each bacterium was confirmed by PCR (Fig. S2A, B). The mutation was complemented with a single copy of *crp* expressed from its own promoter and integrated into the chromosome at the L5 attachment site (Lee *et al.*, 1991). A similar *M. tuberculosis crp* mutant was also generously provided by Dr. R. Buxton, and used in these studies until our new mutant was available. The growth of both *M. tuberculosis crp* and *M. bovis* BCG *crp* mutants was significantly slower than that of their respective WT or complemented strains grown in mycomedium (data not shown), consistent with the phenotype of the *M. tuberculosis crp* mutant described previously (Rickman *et al.*, 2005).

The *serC* and Rv0885 promoter fusion plasmids were transformed into the *M. bovis* BCG *crp* mutant, and their expression was compared in the mutant and WT strains. Deletion of *crp* significantly reduced the expression levels of both *serC* and Rv0885 (Fig. 5B), consistent with the promoter mutation data. CRP_{Mt}'s role in *serC* and Rv0885 expression in *M. tuberculosis* was further confirmed by semi-quantitative RT-PCR using RNA from WT *M. tuberculosis* H37Rv, the *crp* mutant and its complemented strain. Several additional CRP_{Mt} regulon members were included for comparison. Deletion of *crp* reduced the expression of both *serC* and Rv0885 (Fig. 5C). In contrast, expression of another putative CRP_{Mt} regulon member, *lipQ* (Rv2485c), was enhanced in the *crp* mutant. This *lipQ* result is consistent with the finding of a previous report (Rickman *et al.*, 2005). As expected,

expression of Rv0019c, which does not bind with CRP_{Mt} (Bai *et al.*, 2007), was unaffected by *crp* deletion and served as a negative control. Taken together, these data show that CRP_{Mt} directly regulates expression of both *serC* and Rv0885, and that full activation of these promoters is mediated through binding of the previously identified CRP_{Mt} binding site.

***M. tuberculosis serC* rescues the growth defect of an *E. coli serC* mutant**

M. tuberculosis Rv0885 is a conserved hypothetical protein of unknown function, while the predicted product of the divergently expressed *serC* gene is annotated as a phosphoserine aminotransferase (<http://genolist.pasteur.fr/Tuberculist>). However, *M. tuberculosis* SerC has only 18% identity with *E. coli* SerC at the amino acid level, and the function of *M. tuberculosis* SerC has not been confirmed. We cloned the *serC* orfs of *M. tuberculosis* and *E. coli* separately into pMBC664, and expressed each in *E. coli* under control of the *E. coli* *crp* promoter (Fig. 6A, B). Either construct was able to complement a *serC* mutation in *E. coli* (KL285) for growth on minimal medium lacking serine (Fig. 6C), while the KL285(pMBC664) vector-only control strain was unable to grow on serine-free plates (Fig. 6C). This result indicates that the biological function of *M. tuberculosis serC*'s product is similar to that of *E. coli* SerC, so we conclude that *M. tuberculosis* SerC is also a phosphoserine aminotransferase.

Growth of *crp* mutants in supplemented medium

In *E. coli*, SerC is an essential enzyme for the serine and pyridoxine biosynthesis pathways (Fig. 7A) (Man *et al.*, 1997). Our data showed that *serC* expression was significantly decreased in *M. tuberculosis* and *M. bovis* BCG *crp* mutants, so we reasoned that reduced production of serine and/or pyridoxine could be a factor in the slow-growth phenotype of the *crp* mutants. We supplemented mycomedium with 5 mM serine or 5 mM pyridoxine to test the effects of these components on the growth of the *M. bovis* BCG and *M. tuberculosis crp* mutants. Addition of serine accelerated the growth of the mutants ~2 fold during log phase, but addition of pyridoxine was toxic to the mutants. Neither serine nor pyridoxine affected the growth of WT or the complemented mutant strains (Fig. 7B, 7C, S3). These results suggested that the slow-growth of the *crp* mutants was related to the serine biosynthesis pathway.

E. coli SerC catalyzes the interconversion of 3-phospho-hydroxypyruvate and glutamate to 3-phosphoserine and α -ketoglutarate (Man *et al.*, 1997)(Fig. 7A). The 3-phosphoserine is then converted into serine (Man *et al.*, 1997)(Fig. 7A). We tested the effects of these compounds on both *crp* mutants, and found that their growth was significantly increased at mid- to late-log phase by addition of 5 mM phosphoserine, but was not by 5 mM glutamate or α -ketoglutarate (Fig. 7B, C, S3). Because serine is a substrate for biosynthesis of tryptophan, and interconverts with glycine and cysteine (Fig. 7A), we also tested the effects of each of these amino acids on the mutants' growth. Addition of 5 mM glycine or cysteine restored the mutants' growth to a level similar to that with serine present. In contrast, addition of 5 mM tryptophan did not improve growth (Fig. 7B, C, S3).

Complementation of growth defect of *crp* mutants by expression of *serC*

The serine supplementation data suggested that dysregulation of *serC* by *crp* deletion contributed to the mutants' slow growth in vitro. We further tested this possibility using plasmid pMBC1091, which expresses *M. tuberculosis serC* under control of the constitutive *M. tuberculosis tuf* promoter, such that its expression is independent of CRP_{Mt}. Colonies of both the *M. bovis* BCG and *M. tuberculosis crp* mutants harboring pMBC1091 were significantly larger than those harboring the pMBC283 vector-only control (Fig. 8A,B). Constitutive expression of *serC* also restored growth of the recombinant *M. bovis* BCG *crp*

mutant to WT levels by late log phase (Fig. 8C). While constitutive expression of *serC* also improved growth of *M. tuberculosis crp* strain in liquid medium relative to that of the mutant carrying only the vector plasmid (Fig. 8D), it was not to the same extent as was observed on solid media (Fig. 8B), or with direct serine supplementation (Fig. 7B, C). Carriage of the pMBC283 plasmid itself also slowed the mutant's growth, for reasons that are not clear.

The ability of constitutive *serC* overexpression to restore growth of *M. tuberculosis crp* within macrophages was also tested, as the *M. tuberculosis crp* mutant was previously shown to be defective for growth in this environment (Rickman *et al.*, 2005). Macrophages were infected with WT or *M. tuberculosis crp* mutant bacteria that carried either empty vector (pMBC283) or the *serC*-expression vector (pMBC1091). Bacterial growth within macrophages was monitored over a nine-day period by microscopy and plating for colony forming units CFUs (Figure 9). Microscopy was used in parallel with CFUs as an alternative read-out to ensure that the poor plating efficiency of the *crp* mutants at low density did not result in under-counting of mutant bacteria.

As expected, *M. tuberculosis crp* showed little to no growth within macrophages when assayed by either method, while the mutant's growth was fully restored by expression of *crp* in trans. In contrast, constitutive expression of *serC* had no effect on growth *M. tuberculosis crp* within macrophages. Infection levels of the *crp* mutant remained steady throughout a 9 day period, with or without the addition of the constitutive *serC* gene construct. This suggests that bacterial growth, rather than survival is most affected by *crp* deletion. In contrast, the number of WT and *crp*-complemented mutant *M. tuberculosis* bacteria increased significantly over time within macrophages. Thus, the *crp* mutant's intramacrophage defect is not due solely to lack of *serC* expression and its source likely differs from that of its in vitro growth deficiency.

Discussion

Appropriate gene regulation in response to different environmental conditions is critical for *M. tuberculosis*'s pathogenicity during host infection. CRP_{Mt} is a global transcription factor with a putative regulon that includes more than 100 genes (Bai *et al.*, 2005, Krawczyk *et al.*, 2009). We previously showed that the *serC*-Rv0885 intergenic region contains a CRP_{Mt} binding motif that specifically interacts with CRP_{Mt} and CRP_{BCG} in vitro and in vivo (Bai *et al.*, 2007, Bai *et al.*, 2005). However, neither *serC* nor Rv0885 were identified in a microarray study that compared gene expression between an *M. tuberculosis crp* mutant and WT *M. tuberculosis*, raising questions about the role of CRP_{Mt} in their regulation (Rickman *et al.*, 2005). The present study clearly shows that expression of both *serC* and Rv0885 is directly activated by CRP_{Mt} through association with the previously defined CRP_{Mt} binding site (Bai *et al.*, 2005). The absence of these genes from the previous report (Rickman *et al.*, 2005) is most likely due to the sensitivity of the microarray assay or differences in growth conditions between laboratories. Future studies are needed to confirm CRP_{Mt}-mediated regulation of other putative regulon members (Bai *et al.*, 2005).

Regulation of *serC* and Rv0885 by CRP_{Mt} and CRP_{BCG}

In *E. coli*, cAMP is a second messenger within a global regulatory network that includes multiple overlapping regulons that respond to signals such as starvation and anaerobiosis (Kolb *et al.*, 1993). The environmental signals to which CRP_{Mt} responds have not yet been defined. *M. tuberculosis* is also exposed to restricted nutrient conditions and hypoxic environments in the host, and previous studies showed that Rv0885 is up-regulated in both conditions (Bai *et al.*, 2007, Betts *et al.*, 2002, Sherman *et al.*, 2001). However, a direct effect of cAMP levels on Rv0885's regulation has not yet been demonstrated, despite

evidence that CRP_{Mt} (and CRP_{BCG}) allosterically binds this second messenger (Bai *et al.*, 2005, Reddy *et al.*, 2009, Stapleton *et al.*, 2010).

CRP_{Mt} is a homodimer, and cAMP binding reorients the helix-turn-helix domains to maximize specific DNA binding. However, reports differ as to the extent of its asymmetry in the absence of cAMP (Gallagher *et al.*, 2009, Kumar *et al.*, 2010). In contrast with *E. coli* CRP, apoCRP_{Mt} shifts from an open to a closed structure in the presence of cAMP, with the closed structure being the more active one (Reddy *et al.*, 2009). This structural change is consistent with results of an earlier study showing that cAMP binding reduces CRP_{Mt}'s and CRP_{BCG}'s sensitivity to trypsin digestion, while protecting and enhancing their DNA binding ability (Bai *et al.*, 2005, Bai *et al.*, 2007).

Nonetheless, CRP_{Mt}'s cAMP binding affinity and the allosteric effects associated with cAMP binding are relatively low compared with those of *E. coli* CRP (Stapleton *et al.*, 2010), and cAMP is not actually required for at least some of CRP_{Mt}'s DNA binding interactions (Bai *et al.*, 2005, Stapleton *et al.*, 2010). While cAMP levels can be high in mycobacteria, intracytoplasmic levels of cAMP in mycobacteria are similar to those of *E. coli* in many conditions (Bai *et al.*, 2011, Bai *et al.*, 2009, Botsford, 1981, Padh & Venkitasubramanian, 1976, Biville & Guiso, 1985). Thus, the mechanisms by which these mycobacterial CRP orthologs regulate gene expression in response to cAMP may differ from the classical *E. coli* CRP paradigm, particularly with respect to sensitivity thresholds. It is also likely that secondary regulatory factors, such as the repressor predicted from these studies, play important roles as co-regulators, as has been reported for Lrp co-regulation of several CRP-regulated genes in *E. coli*, including *serC* and *serA* (Bilge *et al.*, 1993, Lange *et al.*, 1993, Mathew *et al.*, 1996, Man *et al.*, 1997, Yang *et al.*, 2002). Future studies are clearly needed to address the roles of cAMP levels and co-regulation of CRP_{Mt}-regulated genes in mycobacteria.

Transcription initiation site and gene regulation

Our results indicate that CRP_{Mt} serves as both an activator (*serC* and Rv0885) and, possibly, a repressor (*lipQ*). This study establishes that CRP_{Mt} directly regulates expression of *serC* and Rv0885, but the nature of CRP_{Mt}'s role in *lipQ* regulation has not been determined. cAMP also regulates *serC* expression in *E. coli*, but in this case cAMP-CRP is thought to mediate its regulatory effects indirectly (Lim *et al.*, 1994, Man *et al.*, 1997). In *E. coli*, the position of CRP-binding with respect to the TSS largely determines whether the resulting regulation is positive or negative (Kolb *et al.*, 1993, Botsford & Harman, 1992). CRP-activated promoters tend to have binding sites centered at -41, -61, or -70, placing CRP and the polymerase on the same face of a DNA helix with a periodicity of 10.5 (Kolb *et al.*, 1993). In contrast, CRP binding at other sites (e.g., -60 to +20) typically represses transcription, due to negative interactions with RNA-polymerase (Kolb *et al.*, 1993). The CRP_{Mt} binding site is centered at -55.5 bp from the Rv0885 TSS, so CRP_{Mt}'s activation of Rv0885 expression is consistent with the positive regulation predicted by the *E. coli* CRP model. However, binding of CRP_{Mt} to the site centered at either + 21.5 bp or -12.5 bp from two possible dominant *serC* TSSs also resulted in positive regulation, a result that fits less well with the *E. coli* CRP regulatory paradigm. Nonetheless, the positioning of sites relative to the TSS corresponds well with the 10.5 bp periodicity associated with positive regulation, suggesting that this periodic spacing is a dominant regulatory determinant. Additional minor TSSs are separated by half a helix turn and located even further upstream, at -51.5 or -57.5 relative to the CRP_{Mt} motif. While the relative use of these possible start sites in different conditions remains to be determined, these data suggest that CRP_{Mt}'s binding is not an impediment to RNA polymerase's progression once transcription has been initiated. Detailed regulatory studies are also needed to establish the means by which CRP_{Mt} is able to exert a positive regulatory effect from this unusual position. CRP in *Vibrio cholerae*

regulates expression of CqsA at the level of mRNA stability (Liang *et al.*, 2008), and such additional levels of CRP_{Mt}-mediated control cannot be ruled out in the case of *M. tuberculosis serC*.

DNA sequence substitution of the 'Ctl 1' region, which contains the 16 bp replacement located 18 bp upstream of the CRP_{Mt} motif core for *serC* (Fig. 1D) significantly increased the expression of both *serC* and Rv0885, relative to the expression levels of the WT promoters (Fig. 3). This DNA region is between the TSSs of *serC* and Rv0885, proximal to the -10 sequences of both genes. We propose that an unidentified repressor binds within the 'Ctl 1' region, interfering with RNA polymerase's access to both -10 sequences. This putative repressor is unlikely to be CRP_{Mt}, because the CRP_{Mt}-DNA binding is not changed by the sequence replacement, nor does CRP_{Mt} bind the replacement sequence. Data from another recent study also indicate a high level of complexity in CRP_{Mt}'s regulatory mechanisms (Stapleton *et al.*, 2010). CRP_{Mt}-mediated regulation of *whiB1* expression can be either positive or negative, depending on which of two upstream CRP_{Mt} binding sites is occupied (Agarwal *et al.*, 2006, Stapleton *et al.*, 2010). Additional studies are clearly needed to better define the scope CRP_{Mt}'s regulatory mechanisms for *serC* and other genes.

Effects of CRP_{Mt} on growth of *M. tuberculosis* and *M. bovis* BCG

Our data indicate that CRP_{Mt} regulates expression of *serC*, which encodes a phosphoserine aminotransferase with activity similar to that of *E. coli* SerC. The growth of *M. tuberculosis* and *M. bovis* BCG *crp* mutants is slower than that of their respective WT strains, consistent with CRP_{Mt}-mediated regulation of mycobacterial metabolism. The reported requirement of *serC* for *M. tuberculosis* growth in vitro (Sasseti *et al.*, 2003) led us to test whether decreased levels of *serC* expression in *M. tuberculosis* and *M. bovis* BCG *crp* mutants caused the mutants' slow-growth phenotypes. The improved growth associated with either addition of 5 mM serine or constitutive expression of *serC* from the *tuf* promoter indicates that serine starvation did contribute to the mutants' growth defects. However, *serC* expression restored growth less efficiently than did direct serine supplementation in the case of the *M. tuberculosis crp* mutant. It is possible that higher levels and/or proper regulation of *serC* is required for complete phenotypic complementation, or that *serC* must be co-expressed with other genes in *M. tuberculosis* for the enzyme to be fully effective. In contrast, the ability of *serC* expression alone to fully restore WT levels of growth in the *M. bovis* BCG *crp* mutant indicates that there are metabolic differences between *M. bovis* BCG and *M. tuberculosis* that are at least partly regulated by CRP. Such differences clearly warrant further investigation, and are consistent with previous reports of CRP-associated gene regulatory differences in *M. tuberculosis* and *M. bovis* BCG (Bai *et al.*, 2007, Hunt *et al.*, 2008, Spreadbury *et al.*, 2005).

Serine can be used for biosynthesis of glycine, cysteine, and tryptophan in many organisms (Kitabatake *et al.*, 2000, Largen & Belser, 1975, Pizer, 1965, Ulane & Ogur, 1972). In the present study, addition of glycine and cysteine, but not tryptophan, enhanced the growth of the *crp* mutants at levels similar to those observed with addition of serine. This result supports the proposed biosynthesis pathway (Fig. 7A) in which glycine and cysteine, but not tryptophan, are able to interconvert with serine. The biosynthesis pathways of these three amino acids have not been reported in mycobacteria. However, all of the enzymes necessary to convert serine into these amino acids have been predicted (Table 1). It is likely that a serine bypass exists for tryptophan biosynthesis, or that tryptophan synthesis is sufficient even with low serine levels caused by dysregulation of *serC* in the *crp* mutants.

It is clear that the dysregulation of serine biosynthesis contributes to the growth defect of *crp* mutants in vitro, but restoration of serine biosynthesis by constitutive *serC* expression did not improve growth of the *M. tuberculosis crp* mutant within macrophages (Fig. 9).

Therefore CRP_{Mt}-regulated genes other than *serC* are also critical for *M. tuberculosis* replication within macrophages, and likely in vivo during animal infection. The missing factor(s) could either provide protection against inhibitory components or facilitate metabolic adaptations for growth within the specialized macrophage environment. For an example, we found that the presence of 5 mM pyridoxine (a component of vitamin B6, which exists in vivo) is toxic to *crp* mutants, but not to the WT strains (Fig. 7, S3), and expression of *serC* in the *crp* mutant of *M. bovis* BCG did not reduce this toxicity (Data not shown).

The defective growth of the *crp* mutants with impaired serine biosynthesis suggests that CRP_{Mt} and SerC are potential drug targets for TB therapy. Restoration of in vitro growth by *serC* complementation will facilitate the identification of CRP_{Mt} regulon members that specifically contribute to *M. tuberculosis*'s fitness in vivo, leading to a better understanding of CRP_{Mt}'s role in TB pathogenesis.

Experimental procedures

Bacterial strains and macrophage culture

M. tuberculosis H37Rv and recombinant *M. bovis* BCG (Pasteur strain, Trudeau Institute) were grown in Middlebrook 7H9 medium supplemented with 0.5% glycerol, 10% oleic acid-albumin-dextrose-catalase (OADC), 0.05% Tween-80 (defined as mycomedium), as previously described (Florczyk *et al.*, 2003) or on Middlebrook 7H10 agar (Difco) supplemented with 10% OADC, and 0.01% cycloheximide. Fresh cultures were inoculated from frozen stocks for every experiment. Bacteria were typically used in late log phase after 7 days of growth. Cultures were grown with gentle rocking in ambient air conditions except as specified. *Escherichia coli* strains were grown in Luria broth or Luria agar plates. Kanamycin at 25 µg/ml, or hygromycin at 50 µg/ml, was used where specified. All cultures were grown at 37°C. Addition of cAMP or amino acids is indicated in the Results section for specific experiments.

J774.16 murine macrophage cells were maintained by twice-weekly passages in antibiotic-free tissue culture medium (Dulbecco's modified Eagle's medium (Gibco) supplemented with 5% NCTC (Gibco), 1% nonessential amino acids, 20% fetal bovine serum, and 1% glutamine) as described previously (McDonough & Kress, 1995).

Construction of *M. tuberculosis* and *M. bovis* BCG *crp* mutants

Plasmid p0004S (kindly provided by Dr. William Jacobs, Albert Einstein College of Medicine) (Vilcheze *et al.*, 2008) was modified by digestion with NotI, blunted and religated to generate pMBC819. The PacI-MscI fragment of pMBC819 was further replaced with PacI and MscI digested PCR fragment amplified with KM2271 and KM2257 from pMBC819 to introduce a unique NotI site after PacI and to remove the two PflMI (isochizomer of Van91I) sites within the original PacI-MscI fragment. This construct was designated as pMBC851 and was used to sequentially clone an upstream *crp*-flanking DNA sequence into the newly introduced unique NotI site, and to replace the remaining PflMI fragment with a DNA fragment from downstream of *crp*, to generate pMBC879. We amplified the *M. tuberculosis* *crp* upstream region with KM772 and KM773 (with NotI linkers), and a downstream fragment with KM2061 and KM2062 (with PflMI ends). The final pMBC879 construct was sequence verified before being used to generate *crp* knockouts in *M. tuberculosis* and *M. bovis* BCG, according to published methods (Bardarov *et al.*, 2002, Tufariello *et al.*, 2004). Briefly, pMBC879 was linearized with PacI and ligated with PacI-digested shuttle phasmid phAE159 (kindly provided by Dr. William Jacobs) (Vilcheze *et al.*, 2008). The ligation mixture was packaged using MaxPlax lambda

packaging extracts (Epicentre Biotechnologies) and transduced into *E. coli* HB101. Plasmid DNA was prepared from hygromycin-resistant colonies and transformed into *M. smegmatis* mc²155 to produce the knockout phage stocks. Mutants were screened by PCR with primer pairs KM844 + KM2585 and KM2085 + KM845, which were designed to amplify the junction regions between the insertion marker and flanking chromosomal DNA beyond that which was included in the knockout construct. The disruptions were further confirmed by PCR with primers KM859 + KM860, which amplify internal *M. tuberculosis* *crp* sequences. The *M. bovis* BCG mutant was used throughout these studies, while the corresponding *M. tuberculosis* mutant was used for the macrophage infection experiments. Other *M. tuberculosis* experiments were performed with the *crp* mutant generously provided by Dr. R. Buxton prior to completion of the new *M. tuberculosis* mutant. The deletion endpoints of our *M. tuberculosis* *crp* mutant are identical to those in our *M. bovis* BCG mutant, but differ slightly from those of the mutant generated by the Buxton laboratory. However, the phenotypes of both *M. tuberculosis* *crp* mutants were similar in all assays (not shown).

Complementation of *M. tuberculosis* and *M. bovis* BCG *crp* mutants

M. tuberculosis *crp* was amplified with KM1327 + KM963 and was cloned into the integrating expression vector pMBC409 at HindIII, to generate pMBC587. The *M. bovis* BCG *crp* homolog was amplified with KM1327 + KM963 and cloned into pMBC409 at HindIII site, to generate pMBC1029. Plasmids pMBC587 and pMBC1029 were transformed into *crp* mutants of *M. tuberculosis* and *M. bovis* BCG, respectively, to generate single-copy complemented strains.

serC was expressed in the *crp* mutants using the expression plasmid pMBC283, which contains *oriM* and the *M. tuberculosis* *tuf* promoter, along with both kanamycin and hygromycin resistance markers (from pCRII and pEZ::TNpMOD, respectively). The *serC* orf was amplified using primers KM2713 and 2714 (Table 2), and ligated into pMBC283 at HindIII to generate pMBC1091 for expression of *serC* under control of the *tuf* promoter.

Growth curves of bacterial cultures

Mycomedium was modified by addition of 5 mM of each supplement as specified, and was adjusted to pH 6.6 – 6.8. The growth of WT and *crp* mutants in both *M. tuberculosis* and *M. bovis* BCG was compared in the differentially supplemented media within 25 cm² flasks under gentle shaking conditions, at 37°C with ambient air. Samples of 100 µl were withdrawn and diluted with 100 µl of mycomedium in 96-well plates for each time point. Bacterial concentrations were determined by measurement of the optical densities at 620 nm (OD₆₂₀) using a spectrophotometric microplate reader (Tecan).

Macrophage infections

J774.16 macrophages were seeded at 1×10⁵ cells/ml in 6 well plates approximately 24 h prior to infection, and incubated at 37°C with 5% CO₂. Macrophages were then infected with WT or mutant *M. tuberculosis* strains at a multiplicity of infection of approximately one bacterium per macrophage, as described previously (McDonough & Kress, 1995). Briefly, after 4 h infection, monolayers were washed twice with phosphate-buffered saline (PBS) solution at pH 7.4 and left in fresh tissue culture medium, which was replaced every 2–3 days throughout the infection. At the specified timepoints, macrophages were lysed with 0.5% Triton X-100 and 0.5% sodium deoxycholate for 3 min. Dilutions of cell lysate were then plated onto Middlebrook 7H10 plates and CFUs were enumerated after three weeks for WT and six weeks for mutant strains. For microscopy, infected macrophages were fixed with 4% paraformaldehyde in PBS, stained using a Kinyoun acid-fast protocol ((Kinyoun, 1915), and mounted onto slides for microscopy, as described previously (McDonough & Kress, 1995).

Electrophoretic mobility shift assay (EMSA)

CRP_{Mt} and CRP_{BCG} were expressed and purified as previously described (Bai *et al.*, 2007, Bai *et al.*, 2005). PCR primers were labeled as probes with γ -³²P-ATP using T4 DNA polynucleotide kinase (New England Biolabs). DNA fragments were amplified by PCR, and approximately 0.05 pmol DNA probe was used in each 10 µl of binding reaction, as described previously (Bai *et al.*, 2005). A 500-fold excess of unlabeled DNA was used for competition experiments. Samples were loaded on a non-denaturing 8% polyacrylamide gel and run for 2 – 3 h at 14V/cm. Gels were vacuum dried, exposed on a phosphor screen, scanned with a Storm 860 PhosphorImager (Molecular Dynamics), and analyzed with ImageQuant software.

RNA extraction

Total RNA was extracted using method of Mangan and co-workers (Mangan *et al.*, 1997). After isopropanol precipitation, RNA was treated twice with RNase-free DNase I on a spin column using the RNeasy Mini Kit and RNase-Free DNase Set (Qiagen). The RNA concentration was determined spectrophotometrically with a Biophotometer (Eppendorf) at 260 nm.

Primer extension

The primer extension experiments were performed as described previously (Vasudeva-Rao & McDonough, 2008). [γ -³²P]-ATP labeled primers that were complementary to regions downstream of *serC* (KM1608 and KM1609) and Rv0885 (KM1335) promoters, respectively, were hybridized separately to 20 µg total RNA isolated from exponentially growing *M. bovis* BCG. Hybridization was performed at 100°C for 1 min, followed by annealing for 20 min at 60°C or 65°C for *serC*, or at 52°C for Rv0885. Extension reactions were performed with SSRIII reverse transcriptase (Invitrogen) at 42°C for 1 h. Samples were separated on a 6% polyacrylamide sequencing gel containing 8 M urea. In each case, the adjacent lane contained a DNA sequence ladder generated by dideoxy sequencing (Sanger *et al.*, 1977) of plasmid pMBC648, which contains the *serC*-Rv0885 intergenic sequence, using the relevant oligonucleotide as primer. Gels were vacuum dried, exposed on phosphor screens, and scanned with a Storm 860 PhosphorImager (Molecular Dynamics).

Cloning and mutation of promoters

The intergenic region between *serC* and Rv0885 was PCR-amplified using primers KM1229 and KM1230 (Table 2), and cloned into the single-copy integrative vector, pLACint, upstream of a promoterless *lacZ* gene, as previous reported (Purkayastha *et al.*, 2002). Point mutations and whole motif core substitutions were generated by strand overlap extension (SOEing)-PCR (Horton, 1997, Vasudeva-Rao & McDonough, 2008). KM1229, KM1230, KM1448 and KM1449 were used to generate the G4-to-C mutation. KM1229, KM1230, KM1450 and KM1451 were used to generate the C17-to-G mutation. The motif core sequence (TGTGAGCTGTTTACA) was substituted with a 16 bp random sequence (ATATCTACTGAGTTAC) by using primers KM1229, KM1230, KM1521 and KM1522. A 16 bp sequence (GACACTATGACCCAGC), located 18 bp upstream of the motif core, was substituted with the same random sequence using primers KM1229, KM1230, KM1563 and KM1564 to generate the control-1 DNA fragment. A 16 bp sequence (AGGCTTTTCGTTCCGCT), which is located 18 bp downstream of the motif core, was replaced with the same random sequence by using primer KM1229, KM1230, KM1565 and KM1566 to generate the control-2 DNA fragment. The truncated promoter fragments were amplified using primers KM1463 and KM1230, or KM1464 and Rv1230 for *serC*, and KM1465 and KM1229, or KM1466 and KM1229 for Rv0885. All promoter fragments were

cloned into pLACint at the BamHI site and sequence-verified before being transformed into mycobacteria, to generate the reporter strains.

β-galactosidase activity assay

Bacteria were grown to exponential phase by shaking at 37°C in mycomedium. A 300 µl portion of each sample was transferred to a fresh tube and sonicated, as previously described (McDonough *et al.*, 2000). The β-galactosidase activity of the recombinant cultures was measured as described previously (Vasudeva-Rao & McDonough, 2008) by using 5-acetylaminofluorescein di-β-D-galactopyranoside (C₂FDG) (Molecular Probes), which is cleaved by beta-galactosidase to produce fluorescein. Fluorescein was measured in a CytoFluor Multi-Well Plate Reader (PerSpective Biosystems), and activity readings in arbitrary units were normalized by the optical density of the cultures at 650 nm, using a THERMOmax microplate reader (Molecular Devices). Results were expressed as relative β-galactosidase activity/OD.

Reverse-transcriptase PCR (RT-PCR)

M. tuberculosis WT, *crp* mutant and the complemented strain (kindly provided by Dr. Roger Buxton) (Rickman *et al.*, 2005), were grown to late log phase in mycomedium. cDNA was prepared and amplified as previously described (Gazdik & McDonough, 2005). All samples were standardized against 16S RNA (Alland *et al.*, 1998) using cDNA diluted 10⁻⁴ or 10⁻⁵, and against *sigA* (Manganelli *et al.*, 1999) using cDNA diluted 10⁻². PCR of 16S RNA and *sigA* was also performed prior to cDNA generation, using total RNA without reverse transcription, to ensure the absence of DNA contamination. Gels were scanned with a FluorImager595 (Molecular Dynamics) and analyzed with ImageQuant software (Molecular Dynamics).

Complementation of the *E. coli serC* mutation with *M. tuberculosis serC*

The *E. coli crp* promoter was amplified with primers KM1491 and KM1472 (Table 2), and cloned into pBR322 to generate pMBC664, while the *M. tuberculosis serC*orf was amplified with primers KM2360 and KM2361, and inserted into pMBC664 to generate pMBC886. *E. coli serC* was amplified with primers KM2362 and KM2363, and was cloned into pMBC664 to generate pMBC887. The plasmids pMBC664, pMBC886 and pMBC887 were individually transformed into an *E. coli serC* mutant strain KL285 (CGSC4310) for further analyses (Clarke *et al.*, 1973). Growth among the recombinant strains was compared as described in a previous report (Helgadottir *et al.*, 2007). Briefly, they were grown on M9 minimal medium plates containing 0.2% (w/v) D-glucose and 19 amino acids (excluding serine) at 10 µg/ml each. Serine was added at 50 µg/ml for plates with serine. All plates contained 100 µg/ml ampicillin, and were grown for 48 h at 30°C.

Statistical analysis

A two-tailed *t* test was used to determine the significance of differences between sample results using Prism 4 software. *P* values <0.05 were considered significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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FIG. 1.

Transcriptional initiation sites of *serC* and Rv0885. (A–C) Mapping of the 5' end of Rv0885 with KM1335 (A), and the 5' end of *serC* with KM1609 (B) and KM1608 (C) by primer extension. (D) DNA sequence of *serC*-Rv0885 intergenic region (top strand reads as sense strand for *serC* and antisense strand for Rv0885). The TSS for Rv0885 mapped with Rv1335 is indicated with #. The possible TSSs for *serC* associated with the dominant bands are indicated with * (mapped with KM1609) and with @ (mapped with KM1608), while marks start points that correlate with the additional minor extension product for each primer in the area of the Rv0885 start site. The putative –10 region –10) for each gene is underlined. The translation start codons are shown in boldface. The CRP_{Mt} binding motif and the motif substitution controls 1 and 2 used in later experiments ('Sub Ctl 1' and 'Sub Ctl 2') are indicated with boxes for reference.

FIG. 2.

Transcriptional activity of *serC* and *Rv0885* promoter truncations in *M. bovis* BCG. (A) Schematic of *serC* and *Rv0885* promoter truncations used in *lacZ* reporter constructs. The 'Long' fragment was truncated from the start of the 16 bp motif, while the 'Short' fragment was truncated from the end of the 16 bp motif. The CRP_{Mt} binding site is indicated with filled bars. (B) Expression from *serC* and *Rv0885* promoter truncations compared with expression from their respective full length WT sequences using transcriptional *lacZ* reporter fusions. Activity/OD refers to the amount of beta-galactosidase activity measured in arbitrary units using C2FDG as a substrate per OD₆₅₀ unit of each bacterial culture. Data are presented as mean values $\pm$ standard deviations from three independently repeated biological experiments. *, $p<0.05$, **, $p<0.01$ and ***, $p<0.001$.

FIG. 3. Substitution of the CRP_{Mt} binding motif within the *serC* and Rv0885 promoters. (A) Schematic of the position where a 16 bp sequence within the *serC* and Rv0885 promoters was replaced with 16 bp random sequence (open bar). The binding motif core is shown as a filled bar. Controls were replaced at either 18 bp upstream ('Sub Ctl 1' in Fig. 1), or 18 bp downstream ('Sub Ctl 2' in Fig. 1), of the CRP_{Mt} binding site. (B) Expression of the *serC* and Rv0885 promoters with CRP binding motif substitutions in *M. bovis* BCG, as indicated in panel A. Expression was measured using promoter: lacZ fusions. Activity/OD refers to the amount of beta-galactosidase activity measured in arbitrary units using C2FDG as a substrate per OD₆₅₀ unit of each bacterial culture. Data are presented as mean values $\pm$ standard deviations of three independently repeated biological experiments. *, $0.01 < p < 0.05$. (C) CRP_{Mt} binding with the *serC*-Rv0885 intergenic region and the mutation-containing fragments. The WT and mutation-containing fragments (as shown in panel A) were labeled as probes. CRP_{Mt} was used at 0.7, 1.7, 3.5, 7, 17 nM in the EMSA reactions, shown in order from left to right for each group of six lanes in the figure.

FIG. 4.

Point mutation of the CRP_{Mt} binding motif within *serC* and Rv0885 promoters. (A) EMSA showing binding of CRP_{BCG} with full-length native DNA probe from the *serC*-Rv0885 intergenic region. Lane 1 is probe only; samples in lanes 2 to 7 contain 3.5 nM CRP_{BCG}. Lanes 3 to 7 also include 500-fold excess of unlabeled competitors as follows: 3, 1B5, which is a CRP_{Mt}-binding DNA sequence in Mtb that was identified in a previous study (Bai *et al.*, 2005); 4, a 40 bp DNA sequence upstream of the Rv1624c orf that does not contain a CRP_{Mt}-binding site, which is used as a negative control; 5, native *serC*-Rv0885 motif probe; 6, modified G4-to-C; and 7, modified C17-to-G. (B) Schematic showing positions of point mutations within the *serC* and Rv0885 promoters. Core sequences of the CRP_{Mt} binding motif are represented as a filled block, while the position of each mutation is marked with an asterisk. (C) Effect of the point mutations on transcriptional activity of the *serC* and Rv0885 promoters in *M. bovis* BCG as measured using lacZ fusions. Activity/OD refers to the amount of beta-galactosidase activity measured in arbitrary units using C2FDG as a substrate per OD₆₅₀ unit of each bacterial culture. Data are presented as mean values ± standard deviations of three independently repeated biological experiments. **, $p < 0.01$.

FIG. 5.

Expression of *serC* and Rv0885 in *crp* mutants of *M. tuberculosis* and *M. bovis* BCG. (A) Schematic of *crp* mutant construction. Left-side (Left) and right-side (Right) DNA flanking sequences were cloned into a knockout vector on either side of a hygromycin resistance cassette, for homologous recombination with WT strains. Primers used to confirm the junction in mutants are also indicated. (B) Expression of *serC* and Rv0885 promoter fusions in *M. bovis* BCG WT and *crp* mutant measured using *lacZ* reporter fusions. The promoterless (*lac*) and *tuf* promoter-containing constructs were used as null and constitutive expression controls, respectively. Activity/OD refers to the amount of beta-galactosidase activity measured in arbitrary units using C2FDG as a substrate per OD₆₅₀ unit of each bacterial culture. Error bars denote SD for three repeated experiments; * indicates $p < 0.05$. (C) Regulation of *serC* and Rv0885 by CRP_{Mt} in *M. tuberculosis*. cDNA was synthesized from *M. tuberculosis* WT, *crp* mutant (ko) and its complementary strain (com). The gels shown are representative of three independent experimental repeats. Gels were analyzed using ImageQuant software. The WT/*crp* mutant ratios of the *serC*, Rv0885 and Rv0019c are Means $\pm$ SEM of three independent experiments, which were normalized by *sigA*. *, *lipQ* was only included in one experiment.

FIG. 6. Complementation of *E. coli serC* mutant KL285 with plasmid expressing *serC* from *M. tuberculosis* or *E. coli*. (A and B) PCR confirmation of the presence of *serC* DNA sequences in the appropriate complemented *E. coli* strains, using *serC* primers from *M. tuberculosis* (A), or *E. coli* (B). The amplification of *E. coli crp* was used as a DNA loading control. Lane 1, KL285(pMBC664) control; lane 2, KL285(pMBC886) expressing *M. tuberculosis serC*; lane 3, KL285(pMBC887) expressing *E. coli serC*. (C) Growth of the recombinant strains in the minimal medium with (+ Ser) or without (–Ser) serine. 1, vector-only control strain; 2, *M. tuberculosis serC*-complemented strain; 3, *E. coli serC*-complemented strain.

Fig. 7. Growth of bacterial cultures of WT, *crp* mutant and its complemented strain (Comp) in *M. bovis* BCG with supplemented medium. (A) Serine biosynthesis pathway in *E. coli*. (B) Growth curves of *M. bovis* BCG WT and *crp* mutant in different media. Data shown are representative of two biological repeats. The complemented strain is not distinguishable from WT and is not shown in this panel. (C) Growth of *M. bovis* BCG WT, *crp* mutant and its complemented strain in different media for 7 d. Data shown are means of three experimental repeats. Error bars denote SD. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

Fig. 8.

Constitutive expression of *serC* in *M. bovis* BCG (A and C) and *M. tuberculosis* (B and D) to address the growth defects caused by *crp* deletion. (A and B) Comparison of growth among WT, the *crp* mutant, and each harboring either the promoterless vector control (pMBC283) or vector expressing *serC* under control of the *M. tuberculosis tuf* promoter (pMBC1091). Colonies were imaged after 31 d of incubation at 37°C. (C and D) Growth curves of WT, *crp* mutants, and recombinants expressing of *serC* under *tuf* promoter. Data shown are means of three independently repeated experiments at each time point. Error bars denote SD.

Fig. 9.

Effects of *serC* expression during macrophage infection. Macrophages were infected with WT; the newly generated *crp* deleted *M. tuberculosis* mutant, *crp*, the *crp* mutant carrying a single integrated copy of WT *crp*, com; an empty expression vector, *crp*(pMBC283); or a vector that expresses *serC* from the *tuf* promoter, *crp*(pMBC1091). Infection levels were monitored by plating (A) and microscopy (B) at time points ranging from 4 h to 9 d post infection. Only the WT and *crp* complemented strains showed significant growth within macrophages during this period. The *crp* mutant failed to grow, but the initial infection level was maintained for the full 9 d assay period, and expression of *serC* alone did not restore intramacrophage growth in the absence of *crp*. Data in panel A are the means of two independent experiments, while panel B shows representative images from one of these experiments.

Table 1

Enzymes predicted for serine biosynthesis or for the conversion of serine to other amino acids in *M. tuberculosis*^a

Gene	Rv#	Function	Essential ^b
Serine biosynthesis			
<i>serA1</i>	Rv2996c	Phosphoglycerate dehydrogenase	Y
<i>serA2</i>	Rv0728c	Phosphoglycerate dehydrogenase	N
<i>serB1</i>	Rv0505c	Phosphoserine phosphatase	N
<i>serB2</i>	Rv3042c	Phosphoserine phosphatase	Y
<i>serC</i>	Rv0884c	Phosphoserine aminotransferase	Y
Serine to cysteine conversion			
<i>cysE</i>	Rv2335	Serine acetyltransferase	Slow
<i>cysK1</i>	Rv2334	Cysteine synthase A	N
<i>cysK2</i>	Rv0848	Cysteine synthase A	N
<i>cysM</i>	Rv1336	Cysteine synthase B	N
Serine to glycine conversion			
<i>glyA1</i>	Rv1093	Serine hydroxymethyltransferase 1	N/A
<i>glyA2</i>	Rv0070c	Serine hydroxymethyltransferase 2	N
Serine to tryptophan conversion			
<i>trpA</i>	Rv1613	Tryptophan synthase, subunit	Y
<i>trpB</i>	Rv1612	Tryptophan synthase, subunit	Y

^a All the genes and their functions are predicted (<http://genolist.pasteur.fr/TubercuList/>).

^b Essential (Y) or nonessential (N) by Himar1-based transposon mutagenesis in H37Rv strain (Sassetti *et al.*, 2003). N/A, information not available.

Table 2

Primers used in this study

Primer	Oligo sequence (5' to 3')	Purpose
Disruption of <i>crp</i>		
KM2271	CGTTAATTAAGCGGCCGAGGTCCTGTATCCTAAATC	Construction of pMBC851
KM2257	TCAGCGTTTTGTAATGGCCAGC	
KM772	GCGGCCGCTGGCGTCGTCAAAATTCACC	Upstream homolog fragment
KM773	GCGGCCGCGACCGATCTTGACCTTCC	
KM2061	TTTTTTTTCACAGAGTGAGCGAGCTAGCTTCCTCAG	Downstream homolog fragment
KM2062	TTTTTTTTCACCTTGTGGTCGAGTTGATCGTGGAGGTCG	
KM844	CGCCAAAACCGCTCAACTAGG	Confirm upstream crossover
KM845	CTGGTCGTTGTCGATCCAGG	
KM2085	CTTCACCGATCCGGAGGAAC	Confirm downstream crossover
KM2585	AACGGCAGGTATATGTGATGG	
KM1327	AAGCTTGTCACCGATCTCGATAGCC	Complementation of <i>crp</i> mutants
KM963	AAGCTTGCGTGCGACTCTGTGTCTGC	
Promoter construction^a		
KM1229	GGATCCTATGCGCGTCCTGTCCATTC	<i>serC</i> and Rv0885 promoters
KM1230	GGATCCTGGTCGGCCATGCCATCAG	
KM1448	AACAAGCTCAGATGCAAGCCCTGGCTGGTGG	G4-to-C point mutation
KM1449	GGGCTTGCATCTGAGCTTGTTCACTACGC	
KM1450	GGCGCGTAGTCTGAACAAGCTCACATGCAAG	C17-to-G point mutation
KM1451	AGCTTGTTCAAGTACGCGCCTGTGCCG	
KM1463	GGATCCATGTGAGCTTGTTCACTAC	Truncation of <i>serC</i> promoter
KM1464	GGATCCTACGCGCCTGTGCCGGCAG	
KM1465	GGATCCTGCAAGCCCTGGCTGGTGGC	Truncation of Rv0885 promoter
KM1466	GGATCCAGTGTGAACAAGCTCACATGC	
KM1521	GTAACCTAGTAGATATTGCAAGCCCTGGCTGGTGG	Motif substitution
KM1522	ATATCTACTGAGTTACCTACGCGCCTGTGCCGGCAG	
KM1563	GTAACCTAGTAGATATGCCAAGGGTCTGGATAATTC	Motif substitution control 1
KM1564	ATATCTACTGAGTTACCACCAGCCAGGGCTTGCAATGTG	
KM1565	GTAACCTAGTAGATATGCCGGCACAGGCGCTAGTG	Motif substitution control 2
KM1566	ATATCTACTGAGTTACCAGATCGCCGCGGTTCG	
RT-PCR		
KM859	ACGGCCGAGAAAACCTGTTAACC	<i>crp</i> RT-PCR
KM860	GACCCGCAATGCGCCACCTTCC	
KM886	GCGAACGGGTGAGTAACACG	16S rRNA RT-PCR
KM887	TGAAAGAGGTTTACAACCCG	
KM1276	TCATCTGGTCCGTGTACGG	Rv0019c RT-PCR
KM1277	ATCCTAGATCTTCGACGTACC	
KM1281	CCACCGCTGAGATACGACGC	Rv0885 RT-PCR
KM1282	CGAGTACGTGGAATGCTGG	

Primer	Oligo sequence (5' to 3')	Purpose
KM1286	GGCTATGAGGTGATACTGGG	<i>serC</i> RT-PCR
KM1287	GGCGTCGATGACGACCAAGG	
KM1309	GATGACCGAGCTTAGCGAGC	<i>sigA</i> RT-PCR.
KM1310	CGTAGGTGGAGAACTTGTACC	
KM1496	GGGTGCGAGGATGGTTGTCC	<i>lipQ</i> RT-PCR
KM1497	GTGCGGTAGGTTTCAGCAGCC	
KM1632	CGCCTGTGCCGGCAGGCTT	Verify <i>serC</i> TSS
KM1633	GCCAGGGCTTGATGTGAGC	Verify <i>serC</i> TSS
KM1634	ATCCAGACCCTTGGCGACAC	Verify <i>serC</i> TSS
KM3246	GCCAGGGCTTGATGTGAGC	Verify <i>serC</i> TSS
KM3248	AGCGTTTGCACTTGCTCAAG	Verify <i>serC</i> TSS
KM3275	CCGGGAATTATCCAGACCCTTGGCG	Verify <i>serC</i> TSS
KM3375	GTATCGAACACGGTACCGCTGG	Verify <i>serC</i> TSS
KM3380	ACCCTTGGCGACACTATGAC	Verify <i>serC</i> TSS
Primer extension		
KM1335	GGCGCCACCGCCGAACCTATGC	Rv0885
KM1608	GGTGAGCTGGTCGGCCATGC	<i>serC</i>
KM1609	GCCATCAGGGTAGTGAGGGG	<i>serC</i>
Complementation of <i>serC</i>		
KM2360	GAATTCGCCGACCAGCTCACCCCC	<i>M. tuberculosis serC</i>
KM2361	GAATTCGCACTAAGGCAGCGCTAAAG	
KM2362	GAATTCGGCATTAAACCGTGACGGCG	<i>E. coli serC</i>
KM2363	GAATTCGGCATTAAACCGTGACGGCG	
KM864	GGATATCACACCCGAGGACTACATGGG	<i>M. tuberculosis tuf</i> promoter
KM865	CGGGATCCTTAGTTGGTCCGCTGGAACCTCGC	
KM2713	CGAAGCTTCACTACCCTGATGGCATGG	<i>M. tuberculosis serC</i>
KM2714	CGAAGCTTGCACTAAGGCAGCGCTAAAG	

^aPrimers KM1448 to KM1566 were used in combination with KM1229 or KM1230 to generate promoter mutations of *serC* and Rv0885.

Table 3

Plasmids used in this study

Plasmid	Characteristics	Reference or source
pLACint	Reporter fusion vector	(Vasudeva-Rao & McDonough, 2008)
pMBC391	<i>tuf</i> promoter reporter fusion	(Vasudeva-Rao & McDonough, 2008)
pMBC527	<i>serC</i> promoter reporter fusion	This study
pMBC528	Rv0885 promoter reporter fusion	This study
pMBC577	<i>serC</i> promoter point mutation 1	This study
pMBC578	<i>serC</i> promoter point mutation 2	This study
pMBC579	Rv0885 promoter point mutation 1	This study
pMBC580	Rv0885 promoter point mutation 2	This study
pMBC581	<i>serC</i> promoter deletion 1	This study
pMBC582	<i>serC</i> promoter deletion 2	This study
pMBC583	Rv0885 promoter deletion 1	This study
pMBC584	Rv0885 promoter deletion 2	This study
pMBC622	<i>serC</i> promoter CRP motif substitution	This study
pMBC623	Rv0885 promoter CRP motif substitution	This study
pMBC639	<i>serC</i> promoter CRP motif substitution control 1	This study
pMBC640	<i>serC</i> promoter CRP motif substitution control 2	This study
pMBC641	Rv0885 promoter CRP motif substitution control 1	This study
pMBC642	Rv0885 promoter CRP motif substitution control 2	This study
pBR322		Invitrogen
pMBC664	pBR322 derivative with <i>E. coli crp</i> promoter used as expression vector	This study
pMBC886	pMBC664 derivative to express <i>M. tuberculosis serC</i> in <i>E. coli</i>	This study
pMBC887	pMBC664 derivative to express <i>E. coli serC</i> in <i>E. coli</i>	This study
p0004S	Knockout vector	(Vilcheze <i>et al.</i> , 2008)
phAE159	Knockout phasmid	(Vilcheze <i>et al.</i> , 2008)
pMBC851	P0004S derivative	This study
pMBC879	pMBC851 derivative with <i>M. tuberculosis crp</i> homolog fragments	This study
pMBC914	phAE159 derivative with pMBC879 to knockout <i>M. tuberculosis crp</i>	This study
pMBC409	Integrated vector in mycobacteria	This study
pMBC587	pMBC409 derivative with <i>M. tuberculosis crp</i>	This study
pMBC1029	pMBC409 derivative with <i>M. bovis BCG crp</i>	This study
pMBC283	Expression vector for mycobacteria	This study
pMBC1091	pMBC283 derivative for expression of <i>M. tuberculosis serC</i>	This study